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THE STRATEGY FOR IDENTIFYING FUNCTIONAL ACTIVITIES OF THE PATIENT COMPLEMENT SYSTEM EARLY COMPONENTS LINKED INTO THE BLOOD SUPRAMOLECULAR COMPLEXES OF PATHOLOGICAL ORIGIN: CHEMILUMINESCENT ANALYSIS OF FUNCTIONAL ACTIVITY OF THE C1- INHIBITOR OF PATIENTS WITH THE C4 COMPONENT ISOTYPE DEFICIENCY PROTEIN FACTORS REGULATING THE HUMAN DEFENSE CELLS: INVOLVEMENT OF GLYCOCONJUGATES

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ABSTRACT

A strategy for conducting chemiluminescent analysis (CLA) of the functional activity of the complement early components is proposed. Based on the strategy, CLA has been developed in relation to the C1 inhibitor (C1-inh) of patients with autoimmune diseases and complement C4 component deficiency. The zone pI 4-5 of pathological aggregation of proteins with antigens of the complement components was determined as corresponding to the blotting CLA developed by us to establish the regions of the C4A isotype (enriched mainly with a protein component) and the C4B isotype (more enriched with carbohydrates/ glycans/ glycoconjugates against the background of the presence of protein). The appearance of the C1-inh antigen in the zone indicates its consumption (the appearance of a functional deficiency of the complement component) in the blood with the formation of complex protein and non-protein aggregates with the C1-inh antigen that are not excreted from the bloodstream. In the case of a genetically determined deficiency of C4B in patients (and, as a result, the absence of C4B in the expected area in CLA), the appearance of complexes with C1-inh in the area of C4B location indicates: joint localization of C1-inh and

C4B (linked participation of C1-in with a C4B-dependent complement cascade); the replacement of hereditary C4B deficiency by the participation of C1-inh in C4B-independent cascades of reactions with the result of countering the disease. General prospects for proposed strategy of further using CLA are represented.

KEYWORDS: C1-inhibitor, C4B and C4A, biomarker, functional activity, complement component deficit, isoelectric focusing in a polyacrylamide gel, immunoblotting, Durapore membrane, Immobillon P, chemiluminescence in a live imagination, BioWest, BioChemi System, autoimmune diseases.

ABBREVIATIONS

AFS antiphospholipid syndrome

C1-inh inhibitor of complement component C1 proteinase

C4A isotype C4A of complement component C4, more enriched in protein moiety than carbohydrate moiety

C4B isotype C4B of complement component C4, more enriched in carbohydrate moiety than protein moiety

CLA chemiluminescent analysis

GC glycoconjugates

HCS human complement system

IEF isoelectric focusing

PAG polyacrylamide gel

SLE systemic lupus erythematosus

1. INTRODUCTION

The human complement system (HCS) is an important communicator of innate human immunity, involving relationships with at least 120 molecular registered receptor and soluble protein communicator participants, including those from other known body defense systems.[1] The C1 inhibitor (C1-inh) inhibits/regulates the serine proteinase of the C1 component in HCS - Enzyme Classification (EC) 3.4.21.41-42.[2] C1-inh is a highly glycosylated protein, contains up to 35% carbohydrates by weight. C1-inh, like C4, is an important early regulator of HCS, a clinically significant biomarker of periodic edema, autoimmune and other systemic diseases. Both components of HCS, participating in protective reactions, can be found in covalent assembly immune, autoimmune and glycoconjugate-containing (GC) complexes, including those that are delayed in the blood.

The functionally inactive forms of the complement component that are formed, in general, reduce the whole functional activity of the blood complement component defined as an antigen. The resulting acquired deficiency (a registered decrease in functional activity) of the HCS component negatively affects the entire body's defense system. C1-inh deficiency leads to significant cleavage of the C4 component (as represented by C4A and C4B isotypes) by EC 3.4.21.41-42 serine proteinase forms and an increase in congenital C4 deficiency due to acquired deficiency (deficiency of the C4A or C4B isotype).

With hereditary deficiencies of components, the risk of systemic disorders in the body is increased and expressed.

We have developed promising enzyme immunoassay solid-phase methods for analyzing the functional activity of C4A and C4B isotypes in a micropanel, as well as on a blot - chemiluminescent analysis (CLA) on a blot.[3-7] Since C4 and C1-inh are functionally related in HCS, one of the tasks was to apply CLA in cases of C4 determination to analyze the relative fractional functional activity of C1-inh when considering sera of patients with autoimmune diseases.

The aim is to propose a determination of the relative content of C1-inh (involved in resisting to autoimmune processes in the body) in supramolecular protein complexes and aggregates of the pathological nature in patients' blood based on a universal strategy for the functioning of HCS.

2. MATERIALS AND METHODS

The serums of patients who were examined at the Clinical Diagnostic Center at the G.N. Gabrichevsky Institute were tested. The serums were separated by isoelectrofocusing (IEF, pH 3-5) (600-700 V, 8-10 °C, overnight) in a 5% polyacrylamide gel (PAG) plate. The pH gradient in the gel was controlled (measuring the pH of eluates from gel track fragments, as well as visually using colored markers – *kit pI 2.5-6.5 proteins with methyl red with pI 3.75*; Amersham Pharmacia Biotech, Sweden), as well as sets of recombinant human erythropoietins (non-glycosylated and glycosylated, pI 3-5). The gel was electroblotted onto a *Durapore* (Millipore)—*Immobillon P* (Millipore) membrane sandwich. Blot-pH7 was treated with an acetate buffer pH 4. The determination of C1-inh was carried out on blot-pH4. Deficiency of one of the two C4 isotypes (at a ratio of C4A/C4B or C4B/C4A $\geq$ 2) was confirmed by acidic blotting at pH 2.9. Complement components (C4A and C4B, C1-inh and

C3) were manifested by monospecific antibodies isolated from polyclonal monospecific anti-C4 (or anti-C3, or anti-C1-inh)-serums.

The blots were treated overnight at 4-5 °C (or 3-5 hours at room temperature) on a rocking chair with working dilutions of conjugates of rabbit or goat IgG antibodies to C4 (1: 800-2500), C3 (1: 5000) or C1-inh (1: 1000) human. The proteins on the blot were treated with *SYPRO protein blot stain* (Bio-Rad Lab) dye. Peroxidase was manifested by *BioWest* chemiluminescent substrate (Pierce, USA) with increased sensitivity (superior to ECL and ECL+) and high stability (24 h at room temperature, including in daylight conditions).

Stepwise (sequential nonlinear exposures - seconds, then minutes) kinetics of differentiated intensity were recorded (at each exposure, the recording of the luminous intensity begins from zero, without accumulation/integration of chemiluminescence values at previous exposures) chemiluminescence (—*Bromide Ethydium*‖ light filter). Chemiluminescent paintings were scanned using the *LabWork-4 software package*.

Dye fluorescence (excitation at 254 nm, exposure 30-50 milliseconds) and peroxidase chemiluminescence were recorded in *the Epi Chemi II Darkroom* camera of the *BioChemi System* (UVP Bioimaging Systems, Calif., USA) in live image mode (in real time).

The reprobing CLA (which involves the removal of some superstructures/ assemblies on the sorbed antigen and the reuse of the antigen for chemiluminescent assembly) was performed to comparatively evaluate the binding of antibodies to various complement components on the same blot.

3. RESULTS

3.1. *A strategy for setting a solid-phase analysis of the functional activity of early HCS components* is proposed using examples of glycoprotein C1-inh and glycoprotein isotypes C4A and C4B of the C4 component of the patient blood complement, which were desialylated, isoelectrophoretically separated and electroblotted onto a membranes.

Chemiluminescent solid-phase CLA assumes:

*determination/ identification of the C4A region (mainly protein-rich complexes and aggregates of pI 3.8-4.3 are located; decrease in serum C4A content 415> 408> 400> 392> C1> 395> 393> 399);

*definition/ identification of the C4B region (mainly carbohydrate-rich protein and HA complexes and pI aggregates are located 4.5-4.7; 400> 399> 408> Cu1> 415> 393> 392> 409> 395).

*Identification of antigens of interest in the C4A and C4B regions. Based on the strategy, a CLA of the functional activity of C1-inh (its involvement in the composition of supramolecular aggregates of a pathological nature) in patients with autoimmune diseases characterized by C4A or C4B deficits is proposed (Table 1).

The zone pI 4-5 of pathological aggregation of proteins with antigens of the complement component is determined as corresponding to the CLA procedure developed by us for establishing areas of C4A and C4B isotypes on blots.

Table 1: The severity of C1inh in Blots-pH4 in the isoelectrophoretic regions of C4A and C4B in the serums of patients with a deficiency of the C4A or C4B isotype of C4.

SERA	antiC4-Peroxidase* CLA-blot-pH4				antiC1inh-Peroxidase CLA-pH4			
	C4A	C4B	C4A/C4B	C4B/C4A	C4A	C4B	C4A/C4B	C4B/C4A
sia399								
	76.11	23.89	3.186	0.314	42.43	57.62	0.736	1.359
	73.21	26.79	2.733	0.366	81.89	18.11	4.522	0.221
	55.24	44.76	1.234	0.810	84.42	15.58	5.418	0.185
asia399								
	25.90	74.10	2.857	0.350	64.31	35.69	1.802	0.555
	41.82	58.18	0.719	1.391	-	-	-	-
	28,35	71,65	0,396	2,525	-	-	-	-
In cases of identification of 4B and C1-inh after serum desialylation, the ratio of C4A and C4B regions tended to become the opposite. At the same time, C1-inh was more pronounced in C4A region.								
siaCu1								
	25.35	74.65	0.340	2.941	29.50	70.50	0.418	2.392
	19.46	80.54	0.242	4.132	-	-	-	-
	51.11	48.89	1.045	0.957	38.04	61.96	0.614	1.629
	42.01	57.99	0.724	1.381	-	-	-	-
asiaCu1								
	24.32	75.68	3.115	0.321	54.14	45.86	1.181	0.847
	46.50	53.50	0.869	1.151	-	-	-	-
After desialylation, the proportion of C1-inh was decreased. After desialylation, the proportion of C1-inh was similar in the C4A and C4B regions.								

sia408	69.15	30.85	2.241	0.446	28.70	71.30	0.403	2.481
asia408								
	69.36	30.64	2.264	0.442	42.71	57.29	0.745	1.342
	39.12	60.88	0.643	1.555	-	-	-	-

The changes in the C4A/C4B ratio before and after desialylation were opposite, with C1inh decreasing against the background of an unchanged C4B deficiency.

General comments

*antiC4-Peroxidase, antiC1inh-Peroxidase = peroxidase-labeled antibodies to C 4 or C1-inh. Sia, asia = serum before or after desialylation. Sera of patients with isotype deficiency included Cu1 (patient with SLE), 399 (patient with APS); 408, 415 (patients with APS against the background of existing SLE). Deficiency of the component in the area of C4A (predominance of the protein part in complexes) or C4B (predominance of carbohydrates with proteins, GC in complexes). Isotype deficit was assumed at a ratio of C4A/C4B or C4B/C4A ≥ 2. C4A + C4B = 100%.

The appearance or strengthening of the C1-inh antigen in the C4 isotype zones indicates its consumption and, as a result, the manifestation of a functional deficiency of the HCS component in the blood with the formation of complex protein and non-protein aggregates with the C1-inh antigen that are not excreted from the bloodstream and accumulate in it.

3.2. Details of the CLA procedure for investigation of blotted C1-inh

3.2.1. The selection of serums with a deficiency of one of the isotypes (C4A or C4B) was carried out by our methods of analyzing the functional activity of each of the isotypes in a micropanel using usual non-chemiluminescent dyes, and on a blot using CLA.

3.2.2. Serums were desialyzed by our method using Clostridium perfringens sialidase EC 3.2.1.17. (Grade V, Sigma) under conditions of thermal activation of HCS.

3.2.3. IEF-PAG was performed in the presence of 7M urea (in its absence there is no separation), The application of serums to the gel was carried out in Tween 80 (without tween, proteins remain at the start) in the pH3 region, but not pH4 (an increase in impurities at the start) or pH 6 (there is no clear detection of isotypes).

3.2.4. During blotting, Durapore was used - a hydrophilic membrane gasket that allows free separation of the hydrophobic membrane from the gel; the absence of a membrane led to an

increase in protein impurities in the C4A region, reduced antibody staining; the membrane helped visual assessment of retained protein impurities, was used as a replica of the location of tracks on a hydrophobic immobilizer (according to photographs of semi-dry Durapore in transmitted light in the Epi Chemi II Darkroom camera). The advantages of the blot-pH4-acetate technology (over the blot-pH7) were used in the CLA[6], which gives a more discrete picture of protein bands and takes into account the presence of an acetate anion binding site within the horseradish peroxidase catalytic center, as well as positively tested for major blood glycoproteins C4A and C4B.

3.3. Established relationships between C1-inh and the C4B isotype (Table 1)

3.3.1. C1-inh was predominantly localized in the zone of glycans/GC-containing/binding/forming covalent complexes of serum proteins, characteristic of the location of the C4B – lectin-like isotype with predominant interaction with carbohydrates, glycans and GC, which is capable of forming covalent complexes with them.

3.3.2. The binding of antibodies to C1-inh did not depend on the binding of antibodies to C4 (in serums with C4B deficiency, complexes of antibodies to C1in were found in the area of the missing isotype) or C3 (which affected the area between C4A and C4B, unlike C1-inh). In other cases of patient serums, different targets were observed in the C4B region for antibodies to C4 (at the subisotype level of consideration) or C1-inh.

3.3.3. The content of C1-inh was higher in sera with C4B deficiency when compared with sera with C4A deficiency.

3.3.4. The determination of C1-inh gave the best results in the case of serums with C4B deficiency. At the same time, in some patient serums, the determination of C1-inh activity did not depend much on serum desialation and/or inactivation of serum complement, which could indicate the depth of damage to the body (including as a result of the appearance and expression of the activity of foreign pathogenic sialidase EC 3.2.1.17. in the blood).

4. CONCLUSIONS

4.1. The proposed strategy for determining the functional activity of potentially any component of the HCS is universal.

4.2. The functional activity of the patient's C1-inh serum can be preliminarily evaluated using the proposed CLA on a blot. In this case, it is desirable to compare the result with the analysis of the functional activity of C1-inh in the micropanel with respect to serine proteinases EC 3.4.21.-. in blood.

4.3. The definition of C1-inh is advisable to use for further differentiation of differences in patient sera, as in cases of SLE and APS with a deficiency of the C4B isotype and/or C4A.

4.4. C1-inh is predominantly localized in the aggregation pI region of C4B. This is evident from the presence of glycan/GC-containing supramolecular ensembles with the C4 antigen (the participation of C1-inh in the C4-dependent cascade of HCS is visible).

4.5. The results indicate: a) the systemic depth of damage to the body in SLE combined with APS,

b) a variety of pathways of damage to the body involving C1-inh, c) a potential biomarker of pathological processes. In the future, it is necessary to establish all the causes of inhibitor deficiency- its accumulation in the C4A and (or) C4B regions, due to interaction with serine proteinases EC 3.4.21.-., autoimmune processes, the influence of the highly glycosylated negatively charged part of C1-inh. It is also advisable to reveal the nature of the relationship between the content of C1-inh and a decrease in hemolytic activity in pathologies caused, among other things, by the participation of C4B.

4.6. It should be emphasized that CLA is promising in cases of pathological aggregation electrophoretic regions C4A and C4B, which we have established for the C4 complement isotypes of patients with autoimmune diseases, to assess hereditary and acquired deficiencies of HCS components, including C1-inh.

4.7. The results can also be considered as the potential of CLA for the selection and use of model sera with C4 isotype deficiency and C1-inh deficiency, as well as for the purpose of evaluating the functional activities of other combinations of HCS components.

4.8. SLA is promising as a universal diagnostic tool (linking visual images with specific types of pathologies) for assessing the functional status of the patient's complement, including simultaneous consideration of the contribution of a set of components and activated fragments of HCS components that build a communication network, including taking into account other protective systems of the body.

Disclosure of conflict of interest

The authors declare no conflict of interest.

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